

Cloning of Hamster Type XVII Collagen cDNA, and Pathogenesis of Anti-Type XVII Collagen Antibody and Complement in Hamster Bullous Pemphigoid

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Bullous pemphigoid is an inflammatory subepidermal blistering skin disease associated with an IgG autoimmune response to the type XVII collagen. The immunopathologic features of bullous pemphigoid can be reproduced in mice by the passive transfer of anti-type XVII collagen antibodies. In this model, it is thought that blister formation depends upon complement activation, neutrophil recruitment, and some proteolytic enzymes. In this study, we cloned hamster type XVII collagen cDNA, which contains a 4296 bp coding region and which is predicted to be a transmembrane protein with an extracellular collagenous domain, residing in type II orientation. Antipeptide antibodies (anti-1191 IgG) were obtained against a segment of hamster type XVII collagen homologous with the human type XVII collagen autoantibody-reactive site. The antipeptide antibodies were passively transferred to neonatal Syrian hamsters. The injected hamsters developed a microscopic subepidermal blister as seen

previously in the mice. In order to test whether antigen-antibody complexes and complement initiate the subepidermal blister formation, we carried out experiments *in vitro* on condition that inflammatory cells were completely eliminated. Complement activation in sera was inhibited either by heating (at 56°C for 30 min) or by preincubating with cobra venom factor. When the hamster skin was incubated with fresh anti-1191 antisera, separation of dermal-epidermal junction was observed. The anti-1191 IgG failed to induce C3 deposition and dermal-epidermal junction separation, however, if the anti-1191 IgG was added alone or complement activation in sera was inhibited. Under these conditions, IgG but not C3 was deposited on the basement membrane. These results strongly suggest that antigen-antibody complexes and complement initiate dermal-epidermal junction separation. *Key words: autoimmunity/basement membrane/collagen/skin. J Invest Dermatol 118:485-492, 2002*

Bullous pemphigoid (BP) is an autoimmune bullous dermatosis characterized by subepidermal blisters, a dermal inflammatory infiltrate, and the deposition of autoantibodies and complement along the dermal-epidermal junction (DEJ) (Jordon *et al*, 1967). Ultrastructural studies have shown that the DEJ separation in BP lesions occurs through the lamina lucida, the electron-lucent region that separates the basal cell plasma membrane from the underlying basal lamina (Schaumburg-Lever *et al*, 1972; Dvorak *et al*, 1982). This split is accompanied by an extensive inflammatory infiltrate and the destruction of hemidesmosomal and extracellular matrix components (Schaumburg-Lever *et al*, 1972; Dvorak *et al*, 1982; Anhalt and Morrison, 1993).

One of the main antigenic targets of BP autoantibodies is a 180 kDa hemidesmosome-associated glycoprotein designated as type XVII collagen (also known as BPAG2 or BP 180). It consists of an intracellular domain at its N-terminus, a transmembranous segment, and an extracellular domain (Mutasim *et al*, 1985; Labib *et al*, 1986; Diaz *et al*, 1990; Giudice *et al*, 1991, 1992). The extracellular domain of type XVII collagen contains a series of collagenous domains that is interrupted by 16 (14 in murine) minor noncollagenous (NC) domains at the C-terminus (Giudice *et al*, 1991; Li *et al*, 1993). Structural studies have shown that the type XVII collagen ectodomain exists in a multimeric rod-like conformation (Hirako *et al*, 1996; Balding *et al*, 1997). BP autoantibodies react with at least four distinct antigenic sites on the type XVII collagen ectodomain, all of which are clustered within a 45 amino acid NC stretch in the NC16A domain adjacent to the transmembrane domain (Giudice *et al*, 1993; Zillikens *et al*, 1997).

Liu *et al* (1993) have described a mouse model of BP that involves the passive transfer of antibodies directed against mouse type XVII collagen. Neonatal BALB/c mice injected with these antibodies develop a blistering skin disease that exhibits all of the

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Abbreviations: BP, bullous pemphigoid; CVF, cobra venom factor.

key immunopathologic features of BP. Using this animal model, the researchers have shown that the antibody-induced lesion formation is dependent on complement activation (Liu *et al*, 1995) and the neutrophil infiltration of the upper dermis (Liu *et al*, 1997). The blockage of neutrophil recruitment into skin sites resulted in the neutralization of the pathogenic activity of antimouse type XVII collagen antibodies in mice. Proteinases and reactive free radicals from infiltrating inflammatory cells have been implicated as effector molecules contributing to tissue damage in BP lesions (Jordon *et al*, 1985; Gammon, 1989). Neutrophil granules contain a variety of proteolytic enzymes, including elastase, cathepsin G, collagenase, and gelatinase B, which are known to degrade specific proteins of the extracellular matrix (Senior and Campbell, 1983; Janoff, 1985; Weiss, 1989). Upon cell activation, these enzymes are secreted into the pericellular space (Weiss, 1989). The DEJ separation triggered by pathogenic anti-type XVII collagen IgG could be due to the proteolytic activity of the neutrophil elastase alone or in concert with gelatinase B, cathepsin G, and/or other proteolytic enzymes (Liu *et al*, 2000).

It is possible, however, that the complex of the anti-type XVII collagen IgG and the complement by itself damages epidermal basal cells to trigger clinical blister formation, because the deposition of C3 on the antigen-antibody complexes induces the assembly of the C5b-9 complex (Podack *et al*, 1982). The C5b-9 complex damages basal cells and may initiate blister formation in BP.

We cloned the hamster type XVII collagen cDNA and obtained antipeptide antibodies against the antigenic sites of the molecule. Using the antibodies we investigated the initial step of blister formation *in vitro*. The examination revealed that incubation of the skin caused DEJ separation with the antibodies in the presence but not in the absence of the complement.

MATERIALS AND METHODS

cDNA cloning and sequence analysis We isolated hamster type XVII collagen cDNA with a polymerase chain reaction (PCR) cloning strategy. The total RNA was extracted from neonatal hamster skin (under 24 h old) using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan). The first strand cDNA was reverse transcribed from the RNA using RNA LA PCR kit ver. 1.1 (Takara, Shiga, Japan). PCR amplification of the cDNA was achieved with primers and LA Taq DNA polymerase (Takara). The primers were designed on highly conserved regions of type XVII collagen cDNA of both humans and mice. To extend the nucleotide sequence toward the 5' end of the cDNA, rapid amplification of the cDNA 5' end (RACE) was applied. For the RACE cloning, hamster double strand cDNA with a 5' blunt end was synthesized using DNA synthesis module RPN 1256 (Amersham Life Sciences, Buckinghamshire, U.K.), and the 5' blunt end cDNA was combined with oligonucleotides 5'-GTAATACGACTC-ACTATAGGGCAGCGTGGTTCGACGGCCCGGGCTGGTG-3' and HN-CCCGACCAC-PO4. The adapter-linked cDNA was amplified by PCR using the sense primer 5'-AATACGACTCACTATAGGGC-CGCGTGGTC-3', which binds to the adapter sequence, and the antisense primer, 5'-AGATGCGAGTTCCTTCCGTGGGTACTC-AGG-3'.

Similar strategies were utilized to clone the 3' end of the type XVII collagen cDNA. Specifically, the first round of PCR was carried out with a sense primer, 5'-ATCGGCCAGGAGGAGGTTATG-3' and an antisense primer, 5'-GTTTTCCAGTCACGAC-3' (M13 primer M4; Takara). After this amplification, nested PCR was performed with the nested sense primer oligomer with the sequence 5'-CTGGAGACC-TGGATTACAAC-3' and M13 primer M4 using the prior PCR product as a template. The PCR product was cloned into vector pGEM-T Easy (Promega, Madison, WI) and subjected to DNA sequence analysis.

Chemical synthesis of oligopeptide In human pemphigoid, the 45 amino acid stretch of type XVII collagen contains all the major epitopes

of the molecule (Zillikens *et al*, 1997). We cloned hamster type XVII collagen cDNA and compared the deduced amino acid sequence with that of human type XVII collagen to identify the hamster type XVII collagen epitope. The epitopes, amino acids 495-512 and 513-532, peptides 1190 and 1191, respectively, were synthesized on an Applied Biosystems 430 A peptide synthesizer. The peptides were then purified by using a reverse-phase high performance liquid chromatograph (Applied Biosystems model 150 A) equipped with an Aquapore Prep-10 C-8 column with a linear gradient of 0%-60% acetonitrile in 0.1% (vol/vol) trifluoroacetic acid.

Immunization and immunologic assays Antisera were obtained from young adult rabbits immunized with peptides 1190 and 1191, and were tested for binding with hamster type XVII collagen by immunoblot analysis. They were purified through affinity chromatography on immobilized peptide columns.

Indirect immunofluorescence Sera obtained from immunized rabbits and purified anti-1191 IgG were assayed for antibody titers by indirect immunofluorescence using neonatal hamster skin as substrate (Roscoe *et al*, 1985).

Preparation of hamster skin extracts The neonatal Syrian hamster skin was washed with cold phosphate-buffered saline (PBS) and the dermis was removed as much as possible. The skin was cut into strips and incubated in PBS containing 2 mM ethylenediamine tetraacetic acid and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C for 48 h with one buffer change. The epidermis was separated and extracted according to the method described by Labib *et al* (1986), but with several modifications. Briefly, the epidermis was homogenized on ice for 30 min with 5 ml of 1.5% sodium dodecyl sulfate (SDS), 0.01 M Tris-HCl buffer (pH 6.8) supplemented with 5% 2-mercaptoethanol, 2 mM PMSF (Sigma Chemical, St. Louis, MO), and 10 µg per ml of pepstatin A, antipain, leupeptin, and chymostatin (Sigma Chemical). This was boiled for 5 min and centrifuged at 15,000g for 30 min. The supernatant was harvested and stored at -80°C until use.

Immunoblotting The skin extracts and sera were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions and nonreducing conditions, respectively, according to the method of Laemmli (Laemmli, 1970). Immunoblotting was carried out as described by Towbin *et al* (1992). Antipeptide antisera were diluted 10-fold with TBS buffer.

Administration of the anti-1191 IgG and animal evaluation Neonatal Syrian hamsters (under 24 h old) were injected intraperitoneally with the purified anti-1191 IgG or control IgG. Doses of the anti-1191 IgG ranged from 0.04 to 5.0 mg per g body weight, and the dose of the control IgG was 5.0 mg per g body weight. The hamsters were examined 24 h after the intraperitoneal injection of anti-1191 IgG. The cutaneous change was examined by the extent of epidermis being lifted away from the dermis by tweezers. For histochemical and immunohistochemical studies, the non-fixed hamster skin was frozen in OCT compound. Sections of the skin were incubated with biotinylated anti-rabbit IgG. After the incubation they were washed with PBS and put back into incubation with horseradish-peroxidase-labeled streptavidin. Goat anti-human C3 antibody was found to cross-react with hamster C3 on immunoblotting (data not shown). Therefore, the deposition of C3 was examined with goat antihuman C3 antibody and peroxidase antgoat IgG. This process was followed by a reaction with diaminobenzidine and counterstained with hematoxylin.

Incubation of hamster skin in the immunized serum The skin was obtained from neonatal hamsters (24 h old) and cut into 10 × 10 mm strips with a razor blade. They were incubated with fresh anti-1191 antisera, heat-inactivated (at 56°C for 30 min) fresh anti-1191 antisera, anti-1191 antisera preincubated (at 37°C for 60 min) with 2.5 unit cobra venom factor (CVF) (Venom Supplies, Tanunda, Australia), heat-inactivated fresh anti-1191 antisera plus fresh normal rabbit sera, purified anti-1191 IgG plus Dulbecco's modified Eagle's medium (DMEM), purified anti-1191 IgG plus fresh normal rabbit sera, and purified anti-1191 IgG plus fresh normal rabbit sera preincubated with CVF or control nonimmunized rabbit sera at 37°C for 12 h. All sera and purified

Figure 1. Hamster type XVII collagen cDNA and the deduced amino acid sequence. The deduced amino acid sequence revealed that hamster type XVII collagen consists of a large NC domain (NC-1), amino acids 1-569, and a C-terminal collagenous domain consisting of 14 separate segments with a Gly-X-Y repeating sequence (underlined). The NC-1 domain is predicted to contain the transmembrane domain (amino acids 472-493, bold and underlined). The six potential N-glycosylation sites are shown in bold face.

1 ATGGATGTACCAAGAAAACAGCAGATGGACATGAGTCCACGAGAG 51
1 M D V T K K M K R D G T E V T E R 17

52 ATTGTACAGAAATCTGATACACAGACATCACTCTTACACCAAAAGG 182
18 I V T E I V T T R L T S L P P K G 34

183 AGCAGCAGCAATGATATGTAAGACAGCTCTCTCGCGAGGAGAGTGG 153
35 S T S N G Y A K T G S L G G G S R 51

154 CTAGAGAAACAAAGCTGACATGATGACAGCAGCTACATCAACATG 204
52 L E K Q S L T H G S S G Y I M S S 60

205 GGGAGCATCTGGGAGACGCTTCCACCTCGTTGATAGGAGAGCTCAT 255
69 G S I R G M A S T S S Y R R A H S 85

256 CCGGCTCTCACCTGGCCAACTCTCAGGCTCCACTCTCGAAAGGAAAT 306
88 P A S T L P N S P G S T F E R K T 182

307 CACATGACCTGCTATGAGCTACAGAGGAGCTCAGCGCAACCTCC 357
103 M M T R H G T Y E G S S S G M S S 119

358 CCTGAGTACCAAGGAGAGCTGCTCTCTGAGCAGAGGAGGAGG 400
120 P E Y P R K E L A S S A T R G R S 136

409 CAACACGAGAAAGCAAAATGAGTTGACTGAGAGTGGTCTCTCTC 459
137 Q T R E S E I R V R L Q S A S P S 153

460 ACCAGATGACAGAACTGATGAGTCAAAAGCTTGGTAAAGGAGAGCGA 510
154 T R W T E L D E V K R L L K G S R 170

511 TCTGAGTGGTGGAGTCCACAGGAGACCTCCAGGACCTCCCTCCCTCC 561
121 S A S A S P T R M T S S T L P I P 187

562 AAGAAAGCAGCTGGAGCAAAATGATGACAGAGCTCCCTCCCTCACTA 612
188 K K G T V E T R M Y T A S S H S Y 204

613 TCAGGAGCTATGACAGAGAGCTGAGACCTCACTCTCTCTCTCTCTCT 663
205 S G T Y D T T A L D T N L P S H M 221

664 TGGTCTCTCACTTGGCTGAGGCTCTCTATGAGGAGCTCTCACTCA 714
222 W S S T L P A G S S M G T Y H N M 238

715 ATGACAGCTAGAGCTCATCTCTCTCAAGACCTCTCTCTCTCTCTCTCT 765
239 M T T Q S S S L L M T N A Y S A G 255

766 TCAGTCTCGAAATGCAAAATCACTAGGCTGCTGCTCTCTCTCTCTCTCT 816
256 S V F G M P N M N M A S C S P T L L 272

817 CCGGACTCAGCAGCTGCTCTCTGCTGCTGCTGCTGCTGCTGCTGCTG 867
273 P G L S S C S S V F G M Q M N L A 289

868 CCGAGCTCTGCTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 918
208 P S S S V P A H G G T T T A P T A Y 300

919 GGGGTGAGAAAGAGCTGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 969
307 G V K K N V P Q P P T V T S T G V 323

970 TCCATCT 1020
324 S T S A T C T T S V Q S D B L T L 340

1021 AAGAGCTCTCAAGTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1071
341 K D C K F L I L E K D M N V P S K K 357

1072 GAGATGAGCT 1122
358 F M E L L I M T K D S G K V F T A 374

1123 TCCCT 1173
375 S P A S V S T T S F S E D T L K K 391

1174 GAAAGCAGCT 1224
392 E K Q A A Y A A D A C L K A D I N 408

1225 GAGAGCT 1275
409 G D L N T V S T K G K A T S V E N 425

1276 CATAACTATGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAGCTGAG 1326
426 H N Y D R G G G G S G G G A R G G G 442

1327 GAGAGCT 1377
443 G S G G G G G G G G T W G A P A 459

1378 TGGTCT 1420
460 W C P C G S C C S W W K W L L G L 476

1429 CTGCTCACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1479
477 I L T W L L L L G L L F G L L A L 493

1480 GCGAGAGCT 1530
494 A E V R K L K A R V D E L A T 518

1531 AGGCT 1581
511 R V Q Y F F E D K T E R S S K D R L 527

1582 CTGGGTGATCT 1632
528 L G D M P G V G P G L G R A E L D 544

1633 GCGCAGCT 1683
545 G H S Q E A I P L F V R N K L M T 561

1684 GAACAGCAAAAGCAAAATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1734
562 E Q E N G M L R G M D P P K E D M 578

1735 GAGAGTCAAGGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1785
579 S S Q G P P G D R G L P G T A G T 595

1786 CCGGCT 1836
596 P G P L G H P G P E G P K G Q K G 612

1837 AGCATGAGATCT 1887
613 S I G D P G M E G P I G Q R G L E 629

1888 GCGCT 1938
630 G P M G P R G E P G P P G S G E K 646

1939 GAGAGCAGAGGATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1989
647 G D R G T A G E O G P R G L P G V 663

1990 CCAAGTCT 2040
664 P G S V G P R G S P M G S P G P Q G 680

2041 CCCCAGCT 2091
681 P P G S T G P Q G L R G E V G L E 697

2092 GGTGTCT 2142
698 S V K G D R G L A S P P G P K G S D 714

2143 CAGGCGAGAGAGGAGCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 2193
715 Q G E K G P R G L T G E P G V R G 731

2194 CTACCCTGGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2244
732 L P G A V G E P G A K G A M G P A 748

2245 GCGCT 2295
749 G P D G Q Q G P R G E Q G L T G M 765

2296 CCTGAGCT 2346
766 P G T R G L P G P S G D P G K P P 782

2347 GTCAGAGGCT 2397
783 V T G P Q G P Q G L P G S P G R P 799

2398 GGGACAAAGGCT 2448
800 G T K G E P G A P G R V M T A E G 816

2449 TCATCT 2499
817 S S T I T V P G P P P P G A M G 833

2500 CCCCAGGCT 2550
834 P P G P S G T P G P A G P A G L P 850

2551 GGAACAAAGGCT 2601
851 G Q T K A Q R G E P G L A G D S F 867

2602 ATAGCAGTCT 2652
868 I S S G S S I S E V L A A Q G V D 884

2653 TGGCGGCT 2703
885 L R G P L G P P G P R G P P G P S 901

2704 ATCT 2754
902 I P G P P P G P R G P P G E G V P G 918

2755 CCACT 2805
919 P P G L P G S F L T D S E T F S 935

2806 GCGCT 2856
936 G P P G P P G P P G P K G D Q G D 952

2857 CT 2907
953 P G V P G T P G I P G G H S H G E 969

2908 TCATCT 2958
978 S S S T T Y R Q G P P G P P G P 986

2959 GCGCT 3009
987 G P P G S F S S S G Q D I Q R Y I 1003

3010 CAGAGTCT 3060
1004 A E Y M Q S D S I R T Y L S G V Q 1020

3061 GGTCT 3111
1021 G P P G P P G P P G P P G P I T T I G 1037

3112 GAGCT 3162
1038 E T F D Y S Q L A S Q V V S Y L R 1054

3163 ACATCT 3213
1055 T S G Y G V S L S S A S S E D I L 1071

3214 GCT 3264
1072 A M L R R N D Y W Q F L R Q H L V 1088

3265 GGTCT 3315
1089 G P P G P P G P P G V G S D G S L 1105

3316 CT 3366
1106 L S L D Y G E L S R H I L N Y M S 1122

3367 AGTCT 3417
1123 S S G T S F G H P G P P G P P G 1139

3418 CCAAGTCT 3468
1140 P G T S Y E E L L T N L R G S D Y 1156

3469 AGGAGTCT 3519
1157 R D I I G P P P G P P G P P G P R G 1173

3520 CCCCAGGCT 3570
1174 P P G Y S A A L A T Y A A E N S 1190

3571 AACT 3621
1191 N F R S E L I G Y L T S P D V R S 1207

3622 TTCATCT 3672
1208 F I I G P P G P P G P P G P P G D 1224

3673 AGTCT 3723
1225 S H L R D M Y S W G S S S A R R 1241

3724 GCGCT 3774
1242 G T A Y S S S V G M G A N G S 1258

3775 CTGGGCGAGAGGCT 3825
1259 L G E G R T F G T G D G G P Y G I 1275

3826 GATCT 3876
1276 D T G P G G G Y G A A A G G T Y G 1292

3877 ACCGATCT 3927
1293 T D G D S F R A G F T G D L D Y M 1309

3928 AAGCT 3978
1310 K L A V R V S E S M Q R Q G L L Q 1326

3979 GGGATCT 4029
1327 G M A Y T V Q G P P G P P G P Q G 1343

4030 CCT 4080
1344 P P G I S K Y F S A Y S M V T Q D 1350

4081 CTCATCT 4131
1361 L M D F F R T H G A I P G P P G Q 1377

4132 AAGGAGAGGCGGCT 4182
1378 K S E A G T P G P K G D R G L A G 1394

4183 CAACAGGCT 4233
1395 Q R G P P P G P P G P R G Q K G D K 1431

4234 GGAAGAGAGGCT 4284
1412 G D K G D Q Y V Y T G R R R R S I A 1420

4285 ATCAAGCTATA 4296
1429 I K P + 1432

hamster	1	10	20	30	40	50	50	hamster	951	960	970	980	990	1000	1000
	1	MDVTKKNDK	GTEVTERIVT	EIVTTITLSL	PPKGSSTNGY	AKTGSLLGGGS	50		951	GPKGDQ----	-----	-----	-----	-----	1000
	1	*****	*****	*****	*****	*****	50		951	*****GPPG	PRGHGEGGL	PGFSTSGSSS	FGNLGQPPG	PPGPGPKGD	1000
	1	*****S	*****	*****	*****	*****	50		951	*****	-----	-----	-----	-----	1000
human	1	*****	*****	*****	*****	*****	50	human	951	*****	-----	-----	-----	-----	1000
mouse	1	*****	*****	*****	*****	*****	50	mouse	951	*****	-----	-----	-----	-----	1000
canine	1	*****	*****	*****	*****	*****	50	canine	951	*****	-----	-----	-----	-----	1000
hamster	51	60	70	80	90	100	100	hamster	1001	1010	1020	1030	1040	1050	1050
	51	RLEKQSLTHG	SSGYINSSGS	IRGNASTSY	RRHASPSTL	PNSPGSTFER	100		1001	-GDPVPGTP	GIPGSHSGE	SSSTYRGGP	PGPGPPGPP	GSFSSSGQDI	1050
	51	*****	*****	*****	*****	*****	100		1001	K*****AL	*****P*E*G	*****MV*Q*	*****S*E*	*****E*	1050
	51	*****	*****	*****	*****	*****	100		1001	*****	*****L**A	*****L**P*	*****S*E*	*****E*	1050
human	51	*****	*****	*****	*****	*****	100	human	1001	*****	*****	*****	*****	*****	1050
mouse	51	*****	*****	*****	*****	*****	100	mouse	1001	*****	*****	*****	*****	*****	1050
canine	51	*****	*****	*****	*****	*****	100	canine	1001	*****	*****	*****	*****	*****	1050
hamster	101	110	120	130	140	150	150	hamster	1051	1060	1070	1080	1090	1100	1100
	101	KTHMTRHGT	EGSSSQNSP	EYPRKLASS	ATRGSTQRE	SEIRVQLSA	150		1051	QRYIAEYMS	DSIRTYLSGV	QPPGPPGPP	GPVITITGET	FDYSQLASQV	1100
	101	*****	*****	*****	*****	*****	150		1051	*Q*G*****	*****S*****	*****S*****	*****S*****	*****S*****	1100
	101	*A*****	*****	*****	*****	*****	150		1051	*H*****	*N*****	*****S*****	*****S*****	*****S*****	1100
human	101	*****	*****	*****	*****	*****	150	human	1051	*****	*****	*****	*****	*****	1100
mouse	101	*****	*****	*****	*****	*****	150	mouse	1051	*****	*****	*****	*****	*****	1100
canine	101	*****	*****	*****	*****	*****	150	canine	1051	*****	*****	*****	*****	*****	1100
hamster	151	160	170	180	190	200	200	hamster	1101	1110	1120	1130	1140	1150	1150
	151	SPSTRWELD	EVKRLKGS	SASASPTNT	SSTLPTPKG	TVETKMTAS	200		1101	VSYLRTSGYG	VSL--SSA-S	SEDILAMRR	NDWVFLRQ	LVGPPGPPG	1150
	151	*****	*****	*****	*****	*****	200		1101	*****	*****F*IS-	*****V*Q*	*****S*E*	*****E*	1150
	151	*****	*****	*****	*****	*****	200		1101	*****	*****S*****	*****S*****	*****S*****	*****S*****	1150
human	151	*****	*****	*****	*****	*****	200	human	1101	*****	*****	*****	*****	*****	1150
mouse	151	*****	*****	*****	*****	*****	200	mouse	1101	*****	*****	*****	*****	*****	1150
canine	151	*****	*****	*****	*****	*****	200	canine	1101	*****	*****	*****	*****	*****	1150
hamster	201	210	220	230	240	250	250	hamster	1151	1160	1170	1180	1190	1200	1200
	201	SHSVGTYDT	TALDTNLP	MMSSTLPGS	SMGTYHNM	TQSSSLNTN	250		1151	PGVGGDGLL	SLDYGELSR	ILNYMSSGI	SFGHFGPPG	PGVPGTSYEE	1200
	201	*Q*****	*T**A*****	*****S*****	*****S*****	*****S*****	250		1151	*AS*****	*****A**SR	*****S*****	*****S*****	*****S*****	1200
	201	*****	*****	*****	*****	*****	250		1151	*****	*****	*****	*****	*****	1200
human	201	*****	*****	*****	*****	*****	250	human	1151	*****	*****	*****	*****	*****	1200
mouse	201	*****	*****	*****	*****	*****	250	mouse	1151	*****	*****	*****	*****	*****	1200
canine	201	*****	*****	*****	*****	*****	250	canine	1151	*****	*****	*****	*****	*****	1200
hamster	251	260	270	280	290	300	300	hamster	1201	1210	1220	1230	1240	1250	1250
	251	AYSAGSYFG	PNMMSCSPT	LLPGSLSSS	VFGMQLNAP	SSSVPAHGT	300		1201	LLTMLRGSDY	R-----	-----	-----	-----	1250
	251	*****	*****	*****	*****	*****	300		1201	*****	*****EF	*****V*Q*	*****S*****	*****S*****	1250
	251	*****	*****	*****	*****	*****	300		1201	*****AAGL	S-----	-----	-----	-----	1250
human	251	*****	*****	*****	*****	*****	300	human	1201	*****	*****	*****	*****	*****	1250
mouse	251	*****	*****	*****	*****	*****	300	mouse	1201	*****	*****	*****	*****	*****	1250
canine	251	*****	*****	*****	*****	*****	300	canine	1201	*****	*****	*****	*****	*****	1250
hamster	301	310	320	330	340	350	350	hamster	1251	1260	1270	1280	1290	1300	1300
	301	TAPTATGK	NMPGPTMT	TGVSTATCT	TSVQSDLLH	KDCKFLLEK	350		1251	IGPPGPPGPP	GPRGPGVSA	ALATYAENS	DNFRSELIG	LTSFQVRSFI	1300
	301	*TS*****	*****AAVN	*****A*	*****A*	*****A*	350		1251	*****	*****G	*****S*****	*****S*****	*****S*****	1300
	301	*S*****	*****	*****	*****	*****	350		1251	*****	*****	*****	*****	*****	1300
human	301	*****	*****	*****	*****	*****	350	human	1251	*****	*****	*****	*****	*****	1300
mouse	301	*****	*****	*****	*****	*****	350	mouse	1251	*****	*****	*****	*****	*****	1300
canine	301	*****	*****	*****	*****	*****	350	canine	1251	*****	*****	*****	*****	*****	1300
hamster	351	360	370	380	390	400	400	hamster	1301	1310	1320	1330	1340	1350	1350
	351	DNVPSKIME	LLIMTKDSK	VFTASPVSS	TTSFSEDLK	KEKQAAVAD	400		1301	IGPPGPPGPP	GPRD-----	---SHLRDNY	SWGSSSSARR	GTAVSSVM	1350
	351	*T*****	*****	*****IA	*****	*****N*	400		1301	*****	*****SLLS	TDAA**SSS	*SSH**V**	*SS*****	1350
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mouse	351	*****	*****	*****	*****	*****	400	mouse	1301	*****	*****	*****	*****	*****	1350
canine	351	*****	*****	*****	*****	*****	400	canine	1301	*****	*****	*****	*****	*****	1350
hamster	401	410	420	430	440	450	450	hamster	1351	1360	1370	1380	1390	1400	1400
	401	ACLKADIND	LNTVSTKKA	TSVENNDR	GGSGL--G	GARGGGSGG	450		1351	GGANGSLGE	GRTFG--TGD	GPPYTDIGP	GGGYGAAA-G	GIYTGDSF	1400
	401	SG**EA**	*G*****T	*TADIS**	--SS**---	*GS**--*	450		1351	*GG**EA**	*GA**EA**	*****	*****E*	*MAGNGLL	1400
	401	T*****	*****	*****	*****	*****	450		1351	*****	*****	*****	*****	*****	1400
human	401	*****	*****	*****	*****	*****	450	human	1351	*****	*****	*****	*****	*****	1400
mouse	401	*****	*****	*****	*****	*****	450	mouse	1351	*****	*****	*****	*****	*****	1400
canine	401	*****	*****	*****	*****	*****	450	canine	1351	*****	*****	*****	*****	*****	1400
hamster	451	460	470	480	490	500	500	hamster	1401	1410	1420	1430	1440	1450	1450
	451	GGGGGTGMA	APAKPCGSC	CSMMMLGL	LLTMLLLGL	LFGLIALAE	500		1401	RAGTADLDY	NKLAVRSES	MQRGLLQGM	AYTWGPPGV	PGPGPPGIS	1450
	451	*A*****	*****	*****	*****	*****	500		1401	G*****	*****	*****	*****	*****	1450
	451	*****	*****	*****	*****	*****	500		1401	*****	*****	*****	*****	*****	1450
human	451	*****	*****	*****	*****	*****	500	human	1401	*****	*****	*****	*****	*****	1450
mouse	451	*****	*****	*****	*****	*****	500	mouse	1401	*****	*****	*****	*****	*****	1450
canine	451	*****	*****	*****	*****	*****	500	canine	1401	*****	*****	*****	*****	*****	1450
hamster	501	510	520	530	540	550	550	hamster	1451	1460	1470	1480	1490	1500	1500
	501	VRLKARVDE	LERR--VQY	FEDKTERSK	DRLGDMGV	GPGLGAELO	550		1451	KVFSAYSNT	QDMDFFRTH	GAIPGPPGQ	GEATGPPKG	DRGLAQGRP	1500
	501	*****	*****	*****	*****	*****	550		1451	*****	*****	*****	*****	*****	1500
	501	*****	*****	*****	*****	*****	550		1451	*****	*****	*****	*****	*****	1500
human	501	*****	*****	*****	*****	*****	550	human	1451	*****	*****	*****	*****	*****	1500
mouse	501	*****	*****	*****	*****	*****	550	mouse	1451	*****	*****	*****	*****	*****	1500
canine	501	*****	*****	*****	*****	*****	550	canine	1451	*****	*****	*****	*****	*****	1500
hamster	551	560	570	580	590	600	600	hamster	1501	1510	1520	1530	1540	1550	1550
	551	GHSQKAEIM	VINKLMTQE	NENLRNFGP	KGDMSGQPK	GDRGLPGTAG	600		1501	PGPPGPPGQ	GDKGDKGQV	YTG-RRRRI	AIKP.....	1550
	551	SD**EL**	*K*****	*****S*****	*****S*****	*****S*****	600		1501	*****	*****	*****	*****	*****	1550
	551	*****	*****	*****	*****	*****	600		1501	*****	*****	*****	*****	*****	1550
human	551	*****	*****	*****	*****	*****	600	human	1501	*****	*****	*****	*****	*****	1550
mouse	551	*****	*****	*****	*****	*****	600	mouse	1501	*****	*****	*****	*****	*****	1550
canine	551	*****	*****	*****	*****	*****	600	canine	1501	*****	*****	*****	*****	*****	1550
hamster	601	610	620	630	640	650	650	hamster	1551	1560	1570	1580	1590	1600	1600
	601	IPGRLGHPG	EGPKGKGST	GDPGMEGIG	QRGLEGNIGP	RGEPPGSG	650		1551	*****	*****	*****	*****	*****	1600
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mouse	601	*****	*****	*****	*****	*****	650	mouse	1551	*****	*****	*****	*****	*****	1600
canine	601	*****	*****	*****	*****	*****	650	canine	1551	*****	*****	*****	*****	*****	1600
hamster	651	660	670	680	690	700	700	hamster	1601	1610	1620	1630	1640	1650	1650
	651	EKGQRIAGE	OGPIRLPGV	GSVGPGRNG	SPGPGPPGS	TGPGQLRGEV	700		1601	*****	*****	*****	*****	*****	1650
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	651	*****	*****	*****	*****	*****	700		1601						

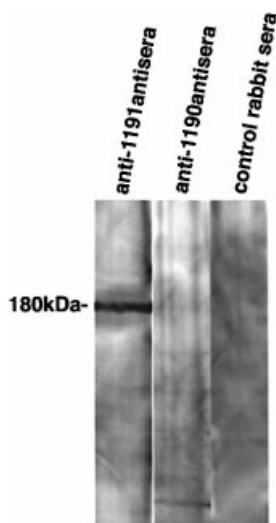


Figure 3. Reactivity of anti-peptide 1191 antisera and anti-peptide 1190 antisera with the 180 kDa protein of hamster skin extracts. Hamster epidermal extract known to contain type XVII collagen was separated on a 7.5% SDS polyacrylamide gel, and transferred to a PVP membrane. The membrane was cut and incubated with rabbit anti-1191 peptide antisera, anti-1190 antisera, or control rabbit sera.

anti-1191 IgG used on incubation with skin strips were reactive up to 1:320 dilution by indirect immunofluorescence. At the end of the incubation, the skin strips were rinsed with PBS and frozen in OCT compound.

RESULTS

The hamster type XVII collagen cDNA sequence and comparison of hamster, human, murine, and canine type XVII collagen amino acid sequence Hamster type XVII collagen cDNA and amino acid sequences are shown in **Fig 1**. The coding region of the cDNA was 4296 nucleotides in length. The nucleotide sequence of the cDNA was submitted to the GenBank database (accession number AB027759). Examination of the 5' nucleotide sequence indicated that the deduced N-terminal region of the type XVII collagen polypeptide consisted of a NC segment of 569 amino acids, designed as the NC-1 domain (**Fig 1**). By using three independent computer programs we predicted the presence of one transmembrane domain within the NC-1 with a cytoplasmic N-terminal (Cserzo *et al*, 1997; Hirokawa *et al*, 1998; Nakai and Horton, 1999). Similarly to that in humans, mice, and canines, hamster type XVII collagen is considered to be a transmembrane protein with type II orientation (Giudice *et al*, 1992; Li *et al*, 1993; Xu *et al*, 2000).

Downstream of NC-1, 14 separate collagenous domains with a characteristic Gly-X-Y repeat were identified. The sizes varied from 9 to 242 amino acids (**Fig 1**). These collagenous domains were separated by various sizes (4–56 amino acids) of NC interruptions. The total number of amino acids in the collagenous domain (570–1432) was 863, representing the C-terminal portion of the molecule. Primary sequence analyses of the deduced hamster type XVII collagen amino acid revealed the presence of six potential N-glycosylation sites (**Fig 1**).

Human and mouse type XVII collagen were shown to consist of 1497 and 1433 amino acids, respectively, and 709 amino acids were deduced from partial canine type XVII collagen cDNA (Giudice *et al*, 1992; Li *et al*, 1993; Xu *et al*, 2000). A direct comparison of the deduced amino acid sequences of hamster, human, mouse, and canine type XVII collagen is depicted in **Fig 2**.

The identities of the total type XVII collagen amino acid sequence of the hamster with those of the human and mouse were

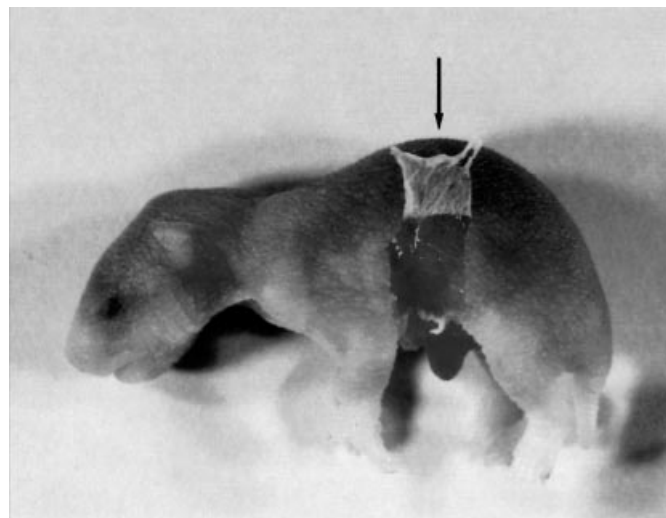


Figure 4. Separation of the DEJ of hamsters administered with anti-1191 IgG. Neonatal hamsters were intraperitoneally injected with IgG at doses from 0.04 to 5.0 mg anti-1191 IgG per g body weight. The epidermis shown in this figure was obtained from a hamster injected with 1.0 mg anti-1191 IgG per g body weight. The epidermis was easily lifted away from the dermis with tweezers 24 h after injection (arrows).

76.4% and 89.4%, respectively, and the identity of the partial amino acid sequence (709 amino acids) of the hamster with the canine type XVII collagen was 82.2%. The antigenic site on the type XVII collagen, however, was not conserved among the hamster, human, mouse, and canine (**Fig 2**). The identities of the hamster with the human, mouse, and canine were 57.5%, 71.8%, and 57.5%, respectively. The amino acid sequences of the transmembranous domain are identical among these species.

Characterization of rabbit antihamster type XVII collagen antisera Reactivities of the rabbit antisera prepared against peptides 1190 and 1191 were characterized by immunoblot analysis. Antisera against peptide 1191 reacted with the 180 kDa protein in hamster skin but anti-peptide 1190 antisera did not (**Fig 3**). Therefore, the anti-1191 IgG (anti-hamster type XVII collagen IgG) was purified by using 1191-peptide columns.

Clinical evaluation of neonatal hamsters injected with anti-1191 IgG Neonatal hamsters were intraperitoneally injected with purified anti-1191 IgG. Twenty-four hours after the injection, the epidermis was lifted away from the dermis with tweezers in all hamsters injected with over 0.2 mg anti-1191 IgG per g body weight (**Fig 4**, arrows; **Table I**). One out of three hamsters injected with 0.04 mg anti-1191 IgG per g body weight showed a separation of the epidermis and dermis. The skin of the hamsters injected with control rabbit IgG did not show any clinical signs or separation in their skin (**Table I**).

Histologic and immunohistochemical studies of the skin of the hamsters injected with anti-1191 IgG Histologic examination revealed a narrow subepidermal cleft formation in the skin of all the hamsters injected with over 0.2 mg anti-1191 IgG per g body weight (**Fig 5a**, arrows). These changes were also evident in one of three hamsters injected with 0.04 mg anti-1191 IgG per g body weight.

In all hamsters injected with the anti-1191 IgG, rabbit IgG was detected on the basement membrane zone (**Fig 5b**, arrows). C3 bound on the basement membrane zone was also observed in all hamsters injected with 0.04 mg anti-1191 IgG per g and 0.2 mg anti-1191 IgG per g body weight (**Fig 5c**, arrows; **Table I**). The skin of hamsters injected with control rabbit IgG did not show any pathogenic alteration (**Fig 5d–f**).

Effects of anti-1191 IgG and complement for DEJ separation *in vitro* In order to examine the effects of anti-1191 IgG and complement for DEJ separation *in vitro*, neonatal hamster skins were incubated under various conditions as described in *Materials and Methods*. Histologic examination of these tissues demonstrated that, for DEJ separation, both anti-1191 IgG and complement were

required. Obviously there was no inflammatory cell attachment to the basement membrane (**Fig 6a, j, m, arrows**). A deposition of IgG was observed in the continual basement membrane zone (**Fig 6b, k, n, arrows**). C3 was mainly detected in the basement membrane zone over the subepidermal cleft (**Fig 6c, l, o, arrows**). In all hamster skin strips incubated with anti-1191 IgG alone, fresh anti-1191 antisera preincubated with C566, and purified anti-1191 IgG plus fresh normal rabbit sera preincubated with C566, a deposition of IgG was observed in the basement membrane zone (**Fig 6e, h, q, arrows**). Neither DEJ separation nor C3 deposition in the basement membrane zone was observed in the skin, however (**Fig 6d, f, g, i, p, r**). Similar results were obtained when the skin strips were incubated with heat-inactivated fresh anti-1191 antisera (data not shown). There was no histologic change in any skin strip incubated with control nonimmunized rabbit sera (data not shown).

Table I. Effects of purified anti-type XVII collagen IgG on DEJ separation

Antibody	Doses (mg per g bw)	N _{SS} /N _I ^a	Deposition of ^b	
			IgG	C3
Anti-hamster type XVII collagen IgG	5.0	2/2	2/2	1/2
	1.0	3/3	3/3	2/3
	0.2	3/3	3/3	3/3
	0.04	1/3	3/3	3/3
Control IgG	5.0	0/2	0/2	0/2

^a(Number of hamsters with skin separation)/(number of hamsters injected).
^bNumerator and denominator represent the number of positive and injected hamsters, respectively.

DISCUSSION

We have cloned hamster type XVII collagen cDNA. The deduced type XVII collagen polypeptide was calculated to have a molecular mass of 145 kDa. It was estimated to be 180 kDa, however, based on the mobility of SDS PAGE (**Fig 3**). This discrepancy could be explained by post-translational modifications as shown in **Fig 2**. It was reported that when antibodies to the mouse type XVII collagen were passively transferred into neonatal BALB/c mice, the

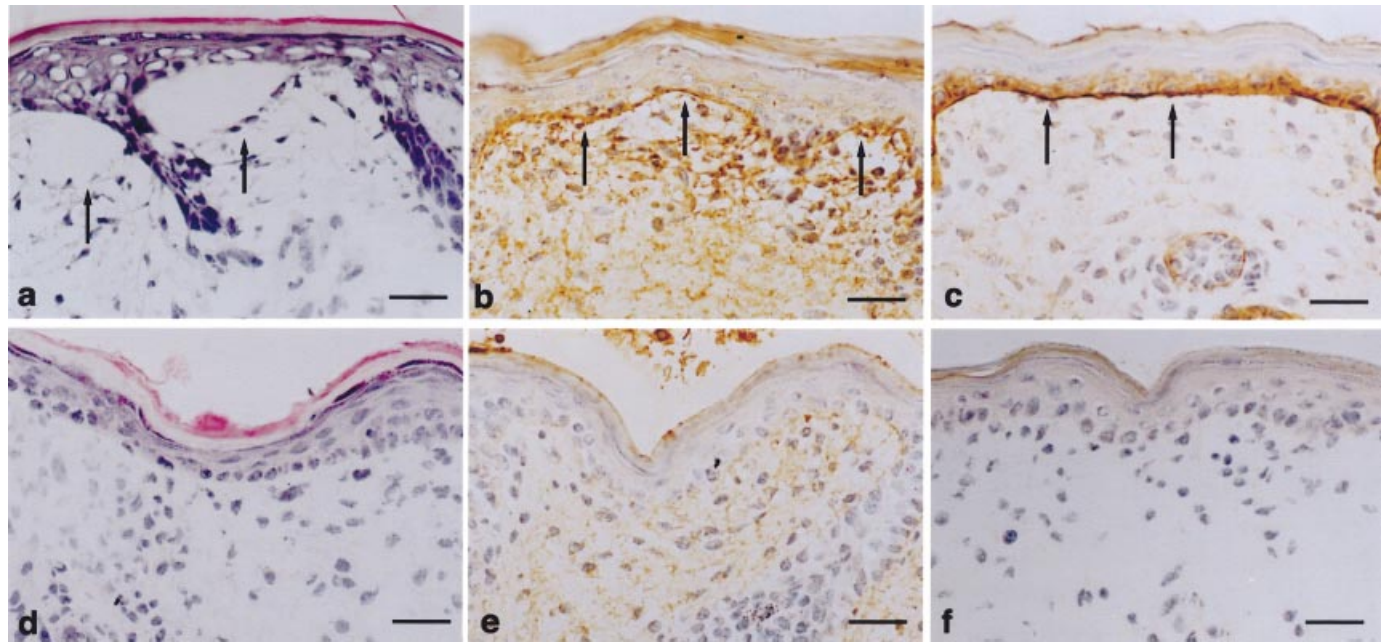
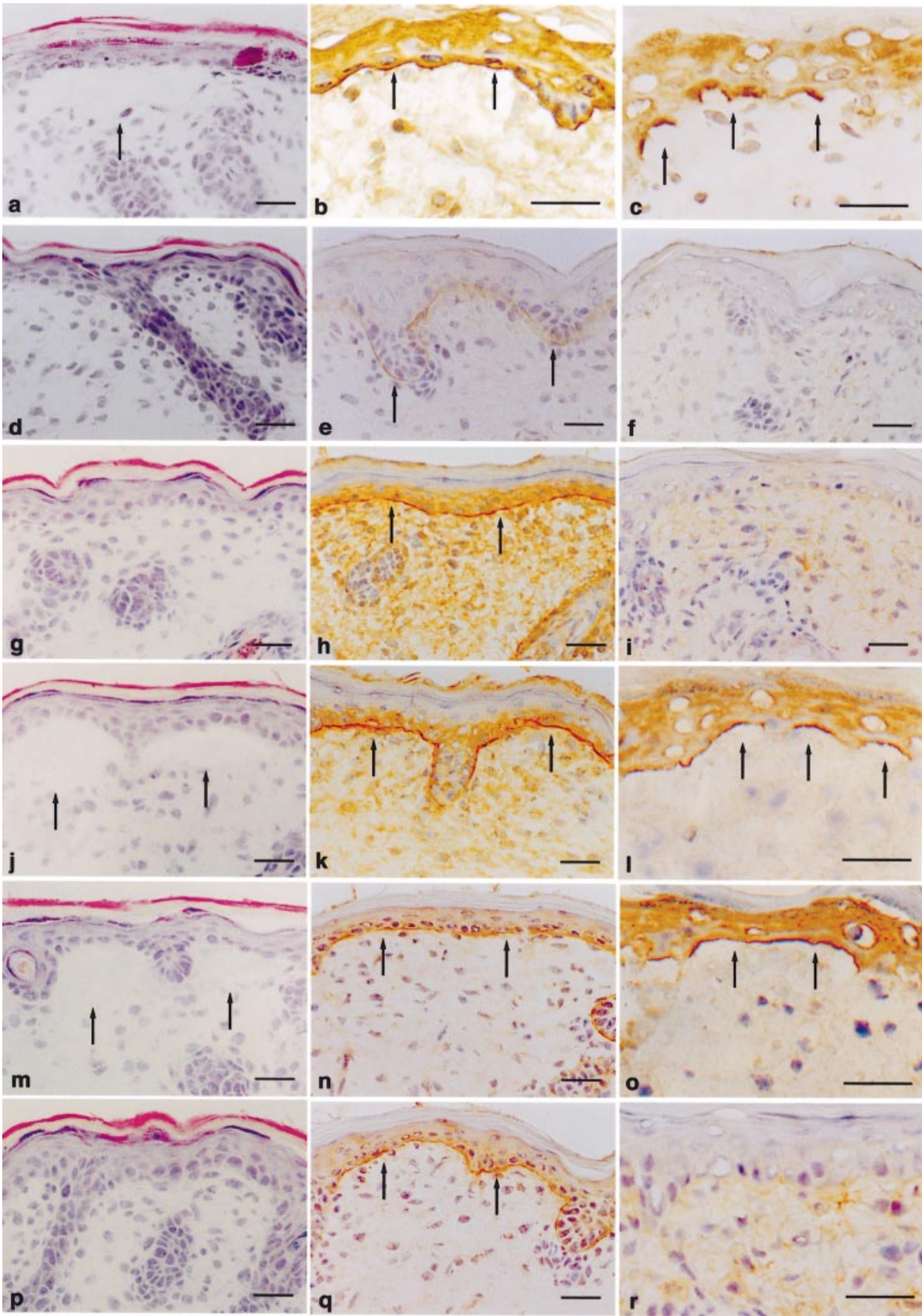


Figure 5. Histologic examination of hamster skin injected with anti-1191 IgG or control IgG. The skins of hamsters injected with 5.0 mg anti-1191 IgG per g body weight (*a-c*) or 5.0 mg control IgG per g body weight (*d-f*) were frozen in OCT compound and sectioned. The sections were stained with hematoxylin and eosin (*a, d*), anti-rabbit IgG (*b, e*), or anti-human C3 (*c, f*). The skins of hamsters injected with anti-1191 IgG showed a subepidermal cleft (*a, arrows*), an accumulation of IgG in the basement membrane zone over a subepidermal cleft (*b, arrows*), and a linear deposition of C3 in the basement membrane zone (*c, arrows*). The skins of hamsters injected with control IgG did not show any pathologic change (*d, e, f*). Neither IgG (*e*) nor C3 (*f*) were observed. Scale bar: 30 μm.

Figure 6. Effects of anti-1191 IgG and complement on DEJ separation *in vitro*. Skins from neonatal hamsters were incubated with fresh anti-1191 antisera (*a-c*), purified anti-1191 IgG plus DMEM medium (*d-f*), fresh anti-1191 antisera preincubated with C566 (*g-i*), heat-inactivated fresh anti-1191 antisera plus fresh normal rabbit serum (*j-l*), purified anti-1191 IgG plus fresh normal rabbit sera (*m-o*), and purified anti-1191 IgG plus fresh normal rabbit sera preincubated with C566 (*p-r*). Sections were stained with hematoxylin and eosin (*a, d, g, j, m, p*), anti-rabbit IgG (*b, e, h, k, n, q*), or anti-human C3 (*c, f, i, l, o, r*). Skins incubated with fresh anti-1191 antisera, heat-inactivated fresh anti-1191 antisera plus fresh normal rabbit sera, and purified anti-1191 IgG plus fresh normal rabbit sera showed a subepidermal cleft (*a, j, m, arrows*), IgG deposition (*b, k, n, arrows*), and C3 deposition in the basement membrane zone over the subepidermal cleft (*c, l, o, arrows*). The skins incubated with anti-1191 IgG, fresh anti-1191 antisera preincubated with C566, and purified anti-1191 IgG plus normal rabbit sera preincubated with C566 showed no pathologic change in the skin (*d, g, p*). Although IgG deposition was observed in the basement membrane zone (*e, h, q*), no C3 deposition was detected (*f, i, r*). Scale bar: 30 μm (*a, d-k, m, n, p, q*), 12 μm (*b, c, l, o, r*).

antibodies induced a cutaneous blistering disease that reproduced all of the clinical and immunopathologic features observed in human BP (Liu *et al*, 1993). This DEJ separation triggered by pathogenic

antitype XVII collagen IgG could be due to the proteolytic enzymes activated by complement and neutrophil recruitment (Liu *et al*, 2000). In order to examine the DEJ separation mechanism in



hamster BP, we obtained anti-hamster type XVII collagen antibodies by injecting into rabbits two peptides (1190 and 1191) in the antigenic site of the molecule. As only anti-1191 antisera were found to react with hamster type XVII collagen, anti-1191 IgG was purified from the antisera and used for pathogenic studies of hamster BP. In neonatal hamsters injected with anti-1191 IgG, the epidermis was lifted away from the dermis by tweezers. Histologic examination demonstrated a narrow subepidermal cleft formation of the skin in all hamsters injected with over 0.2 mg anti-1191 IgG per g body weight. IgG was found to bind to the basement membrane zone in all the anti-1191 IgG-injected hamsters. C3 deposition in the basement membrane zone was always detected in hamsters injected with low doses of anti-1191 IgG (0.04–0.2 mg per g body weight), but was not always seen in hamsters injected with higher doses of anti-1191 IgG (1.0–5.0 mg per g body weight). The higher doses of anti-1191 IgG and complement produced more extensive damage to the basement membrane and might destruct C3 in the basement membrane zone. The contribution of neutrophils to the DEJ separation was reported by using the *in vitro* system (Gammon *et al*, 1982). This showed that when one of the leukocytes, pemphigoid antibodies, or fresh human serum was omitted, significantly less basement membrane zone separation occurred.

We tested the possibility that antigen–antibody complexes and complement components may initiate the DEJ separation. In order to test this hypothesis, we incubated hamster skin with anti-1191 IgG in combination with sera containing active or inactive complement system. In order to inactivate the complement system, fresh anti-1191 antisera and fresh normal rabbit sera were either inactivated with heat (at 56°C for 30 min) or preincubated with CVF (at 37°C for 60 min). BP-like DEJ separation was observed exclusively when the skin was incubated with anti-1191 IgG in sera containing active complement system. Under these conditions, both IgG and C3 were deposited on the basement membrane zone. In the skin incubated with anti-1191 IgG alone, IgG but not C3 was deposited on the basement membrane zone; therefore DEJ separation was not observed. An addition of sera whose complement system was inactivated with heat or CVF to anti-1191 IgG caused IgG deposition, but neither C3 deposition on the basement membrane zone nor epidermis–dermis detachment. It was shown in these studies that both the anti-1191 IgG and complement system were essential for DEJ separation. The anti-1191 IgG and type XVII collagen complex were thought to activate the classical pathway of complement in the sera, because C3 was deposited in the basement membrane zone. Activated C3, in turn, produces the terminal C5b-9 complement components on the basement membrane zone to cause DEJ separation. It is likely that this DEJ separation by anti-1191 IgG and complement is the initial step of clinical blister formation, and proteolytic enzymes secreted by neutrophils may work to enlarge the separation and blister formation.

In summary, this study showed that rabbit antipeptide antibody against the hamster counterpart of the human pemphigoid antigen decreased dermal–epidermal cohesion and induced DEJ separation. Histologic and immunohistochemical examination demonstrated a narrow subepidermal cleft formation, and a deposition of IgG and C3 in the basement membrane zone without leukocyte infiltration. Anti-hamster type XVII collagen IgG and complement started DEJ separation in the absence of inflammatory cells. The decrease in dermal–epidermal cohesion due to the IgG and complement appears to play a key role in the pathogenic cascade leading to blister formation.

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